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Title: Inhibition of the interaction between protein phosphatase 1 glycogen-targeting subunit and glycogen phosphorylase increases glycogen synthesis in primary rat hepatocytes

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Abbreviations: GP, glycogen phosphorylase; GPi(s), glycogen phosphorylase inhibitor(s); GS, glycogen synthase; PP1, type I protein phosphatase; G_L , liver isoform of PP1-glycogen targeting subunit; GP α , active form of glycogen phosphorylase; GS α , active form of glycogen synthase; G_L -GPi, inhibitor of the G_L -GP interaction; hGP, human liver glycogen phosphorylase

SUMMARY

In type 2 diabetes, increased glycogenolysis contributes to the hyperglycaemic state, therefore the inhibition of glycogen phosphorylase (GP), a key glycogenolytic enzyme, is one of the possibilities to lower plasma glucose levels. Following this strategy, a number of GP inhibitors (GPis) have been described. However, certain critical issues are associated with their mode of action, e. g. an impairment of muscle function. The interaction between GP and the liver glycogen targeting subunit (G_L) of protein phosphatase 1 (PP1) has emerged as a new potential anti-diabetic target, as the disruption of this interaction should increase glycogen synthesis, potentially providing an alternative approach to counteract the enhanced glycogenolysis without inhibiting GP activity. We identified an inhibitor of the G_L -GP interaction (G_L -GPi) and characterized its mechanism of action in comparison to direct GPis. In primary rat hepatocytes, at elevated glucose levels, the G_L -GPi increased glycogen synthesis similarly to direct GPis. Direct GPis significantly reduced the cellular GP activity, caused a dephosphorylation of the enzyme and decreased the amounts of GP in the glycogen-enriched fraction; the G_L -GPi did not influence any of these parameters. Both mechanisms increased glycogen accumulation at elevated glucose levels. However at low glucose levels, only direct GPis led to increased glycogen amounts, whereas the G_L -GPi allowed the mobilization of glycogen because it did not block the activity of GP. Due to this characteristic, G_L -GPi in comparison to GPis could offer an advantageous risk-benefit profile circumventing the potential downsides of a complete prevention of glycogen breakdown while retaining glucose lowering efficacy, suggesting that inhibition of the G_L -GP interaction may provide an attractive novel approach for rebalancing the disturbed glycogen metabolism in diabetic patients.

1. INTRODUCTION

Type 2 diabetes is a polygenic disease characterized by peripheral insulin resistance, elevated hepatic glucose production and defects in pancreatic insulin secretion [1]. The relative importance of each of these abnormalities for the aetiology and progression of type 2 diabetes is not fully established to date. However, it is generally accepted that excessive glucose production by the liver contributes significantly to the diabetic hyperglycaemia [2-4]. In the diabetic liver, glucose production is increased, in part, due to the elevated glycogenolysis as a consequence of the enhanced activity of GP. Therefore, the blockade of glycogenolysis via the inhibition of GP has been proposed as one of the therapeutic strategies to improve the glycaemic control in type 2 diabetes.

A number of GPis with different GP binding sites and hence distinct profiles with respect to potency, kinetics, synergism with glucose, and ability to promote the dephosphorylation of GP have been described [5]. However, in spite of the variety of GPis discovered, certain critical issues remain associated with a direct inhibition of the GP enzyme as an anti-diabetic strategy. Among these are the potential risks of hypoglycaemia and potentially negative impact on muscle function. Foreseeing the latter one is supported, first, by a study showing that the pharmacological inhibition of GP in a perfused muscle system impairs the aerobic muscle function during prolonged contraction [6]. And second, the exercise capacity is impaired in patients with McArdle's disease, subtype V of glycogen-storage diseases caused by GP deficiency in muscle [7, 8]. Therefore, alternative strategies for the correction of glucose disposal would be of interest.

In recent years, the interaction between GP and the liver glycogen targeting subunit (G_L) of protein phosphatase 1 (PP1) has emerged as a potential novel target for the treatment of type 2 diabetes, as the G_L -GP interaction plays an important role in the allosteric regulation of glycogen synthesis [9]. Glycogen synthesis itself is regulated via changes in the phosphorylation state of glycogen synthase (GS). The key regulator of the phosphorylation state of GS is PP1 which dephosphorylates the enzyme and brings it to the active state (GS_a). In the liver, targeting of PP1 to glycogen particles by G_L increases PP1 activity towards GS [10, 11]. However, apart from targeting PP1 to glycogen, G_L also interacts by its C-terminus with the active form of GP (GP_a). This interaction suppresses the activity of PP1 towards GS and thereby inhibits glycogen synthesis [12]. This allosteric regulation of PP1 by GP_a allows glycogen synthesis to be switched off when the glycogenolytic pathway is active. However, in the diabetic liver, increased conversion of GP to its active (a) form sustains the allosteric inhibition of glycogen synthesis, furthermore depleting glycogen pools and contributing to the elevated glucose production. Under these circumstances, an inhibition of the G_L -GP interaction would be of particular relevance as an anti-diabetic strategy as it would help to counteract the abnormally increased hepatic glucose output. However, no compounds working via this novel mechanism have been described so far.

In the present study, following the identification and initial characterization of a G_L -GP inhibitor (G_L -GPI), the effects of this tool compound on glycogen metabolism were analyzed in comparison with two direct GP inhibitors. Using primary rat hepatocytes, we demonstrate here that the pharmacological prevention of the G_L -GP interaction increases both glycogen synthesis rate and glycogen amounts at elevated glucose levels similarly to direct GPis, however without inhibiting the activity of GP.

2. EXPERIMENTAL

Isolation and culture of primary rat hepatocytes

Primary hepatocytes were isolated by collagenase perfusion from the livers of male Wistar [CrI:Wi(Han)] rats weighting 200-300 g (Charles River, Sulzfeld, Germany). The cells were suspended in William's medium E (Invitrogen, Karlsruhe, Germany) supplemented with 5% (v/v) foetal calf serum, 17 nM insulin (Invitrogen), 100 nM dexamethasone and 2 mM glutamine (Sigma, Taufkirchen, Germany), 0.1 mg/ml gentamicin (Cambrex BioScience, Walkersville, MD) and seeded at 1.1×10^4 or 1.1×10^5 cells per well in 96- or 6-well collagen I-coated plates (Becton Dickinson Labware, Bedford, MA), respectively. After an attachment phase of 3 h, the medium was replaced by serum-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5 mM glucose and other additives as described above (unless otherwise indicated). The cells were incubated overnight (16-18 h) prior to compound treatments.

Compounds

G_L-GPi was identified in a scintillation proximity assay (Amersham Biosciences, Inc) using recombinant [³³P]radiolabelled human liver GP α and a biotin-labelled G_L peptide composed of 17 C-terminal amino acids (²⁶⁹FPEWPSYLG₁YEKLG₂PYY²⁸⁴, (Interactiva, Ulm, Germany)). A selectivity screen performed at MDS Pharma gave no hints on off-target effects (data not shown). Two direct GPis were used as reference compounds (CP-320,626 [13] and compound A, representing Bsp.1 from the Aventis patent [Aventis Pharma Deutschland GmbH: DE 10335092 B3 (February 3, 2005)]).

Compound treatments

Following the exchange of the overnight culture medium to serum-free DMEM containing 2.5 mM or 10 mM glucose and additives as described above, the cells were incubated with compounds for 2 or 6 h. Cells treated with 0.25% (v/v) DMSO served as solvent controls. To stop ongoing biological processes, the cells were washed with ice-cold PBS and immediately stored at -80°C until further usage.

GP activity measurement

Enzymatic activity of GP was measured in the glycogenolytic direction using a phosphoglucomutase/glucose-6-phosphate dehydrogenase-coupled assay. For this, hepatocytes were cultured in 6-well plates and scraped in lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100 and protease inhibitor mix (Roche Diagnostics, Mannheim, Germany) followed by sonication. The reaction mixture contained 20 μ g of protein lysates, 50 mM KH₂PO₄, pH 6.8, 1.3 mM MgCl₂, 100 μ M EDTA, 430 μ M NADP, 9 μ M glucose 1,6-bisphosphate, 0.48 U/ml each glucose-6-phosphate dehydrogenase and phosphoglucomutase, and 4 mg/ml glycogen. The rate of enzyme-catalyzed reaction was monitored at 30°C following the formation of NADPH by absorbance measurements at 340 nm.

Expression and purification of mouse liver GS

HEK293 cells grown on 10 cm culture dishes were co-transfected with pCMV5-GYS2 and EBG6P-Glycogenin expression constructs, coding for mouse liver GS and GST-tagged human glycogenin 1. The transfection was carried out using Lipofectamine 2000 transfection reagent according to the standard protocol (Invitrogen). After 36 h, cells were lysed in 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 10 mM EDTA, 1% (v/v) Triton X-100, 1 mM Na₃VO₄, 50 mM NaF, 5 mM Na₄P₂O₇, 0.27 M sucrose, 0.1% (v/v) β -mercaptoethanol and protease inhibitor mix (600 μ l lysis buffer/plate). Cell lysates were combined and centrifuged at 4 000 g for 10 min at 4°C, afterwards the supernatant was incubated with glutathione-sepharose (Amersham) (1.33 ml 50% beads in lysis buffer per 10 ml lysate) for 1 h at 4°C using an end-over-end

rotator. The GS/GST-glycogenin complexes caught by glutathione-sepharose were recovered by centrifugation at 500 *g* for 5 min at 4°C and washed sequentially with washing buffer 1 (lysis buffer supplemented with 154 mM NaCl) and washing buffer 2 (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM EGTA) twice each. GS bound to GST-glycogenin was eluted with elution buffer (washing buffer 2 supplemented with 20 mM reduced glutathione) for 10 min at room temperature. The protein concentration in the eluate was determined according to the Bradford method.

Dephosphorylation of the recombinant mouse liver GS

Mouse liver GS was suspended in the reaction buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM DTT, 0.1 mM EGTA, 0.025% Tween 20) to the final concentration of 1.25 µg/ml. The dephosphorylation of GS with catalytic subunit of the α -isoform of rabbit recombinant PP1 (25 U/ml final concentration) (Sigma) was performed for 30 min at 30°C in the absence or presence of 17 mM Na₃VO₄. The reaction was terminated by adding protein loading buffer (Roth, Karlsruhe, Germany) and the samples were analyzed by immunoblotting.

GS activity assay

To measure GS activity, hepatocytes were cultured in 6-well plates and lysed in 20 mM Tris HCl, pH 7.4, 50 mM NaF, 5 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) β -mercaptoethanol, 1% (v/v) Triton X-100 and protease inhibitor mix. After a centrifugation step (13 000 *g*, 10 min, 4°C), aliquots of the supernatants (15 µl/reaction at 1.5 mg/ml protein) were suspended in the assay mix (30 µl/reaction) composed of 50 mM Tris HCl, pH 7.4, 25 mM NaF, 20 mM EDTA, 1 mg/ml glycogen, 0.4 mM UDP-glucose, in the presence of either 0.1 mM or 10 mM glucose-6-phosphate. 0.075 µCi of [¹⁴C]UDP-glucose was added to each well to obtain a final specific activity of 20.3 mCi/mmol. After a 30 min incubation at 37°C, glycogen precipitation (2h, 4°C) and liquid scintillation counting were performed. The ratio of activities at 0.1 mM (active GS) and 10 mM glucose-6-phosphate (total GS) was used to determine the degree of GS activation.

To measure the activity of mouse liver GS, the recombinant protein was suspended in the reaction buffer (composition as described above) to the final concentration of 0.03 mg/ml and pre-incubated with the G_L-GPi (0.3-100 µM) or the catalytic subunit of the α -isoform of rabbit recombinant PP1 (147 U/ml final concentration) for 30 min at 30°C. Aliquots (15 µl) from each reaction were assayed for GS enzymatic activity as described above for the cellular lysates.

Preparation of glycogen-enriched fractions

Cell lysates obtained as described for the determination of GP activity, were sequentially centrifuged for 10 min at 1 000 *g* and 15 min at 10 000 *g*, 4°C. The resulting supernatants were further centrifuged at 100 000 *g* (40 min, 4°C). The pellets obtained in the last step were referred to as the glycogen-enriched fraction. They were resuspended in detergent-free lysis buffer, the protein concentrations in the samples were determined according to the Bradford method.

Immunoblotting

10 µg protein/well of cell lysates or glycogen-enriched fractions was electrophoretically separated by SDS-PAGE and transferred onto nitrocellulose membranes according to standard procedures. The membranes were blocked in Tris-buffered saline containing 0.25% (v/v) Tween 20 (TBST) and 5% (w/v) non-fat dry milk and further incubated with antibodies diluted in TBST containing 0.5% (w/v) milk. The primary antibodies were polyclonal anti-GP (Acris Antibodies, Hiddenhausen, Germany), anti-phospho(Ser¹⁴)-GP, anti-GS, anti-phospho(Ser⁶⁴¹)-GS (Cell Signaling Technology, Beverly, MA), all used at 1:1000 dilution, and monoclonal anti- γ -tubulin (1:5000) (Sigma). Following the incubation with the respective

HRP-labelled secondary antibodies, the blots were developed with a Lumi-LightPLUS chemiluminescent substrate (Roche Diagnostics) and signals were detected using a Fuji LAS 3000 Imaging station (Fuji Photo Film Co., Tokyo, Japan).

Glycogen synthesis assay

To determine the rate of glycogen synthesis, primary rat hepatocytes were cultured in 96-well plates for overnight in serum-free William's medium E supplemented as described above. After washing with PBS (37°C), the medium was changed to Krebs-Ringer bicarbonate HEPES buffer containing 10 mM glucose and 0.1% (w/v) BSA. Compounds were applied using a concentration range from 0.01 to 10 μ M and 0.5 μ Ci of [¹⁴C]glucose was added to each well to obtain a final specific activity of 11.2 mCi/mmol. After 2 h incubation at 37°C, the cells were lysed with 1 M NaOH (100 μ l/well), whereupon the lysates were transferred to the glass fibre filter plates (Millipore) pre-loaded with 100% ethanol (100 μ l/well) in order to precipitate the glycogen for 2 h at 4°C. The incorporation of [¹⁴C]glucose into glycogen was measured by liquid scintillation counting.

Glycogen hydrolysis and glucose determination

For the determination of glycogen levels, aliquots (10 μ g) of the glycogen-enriched fractions were used for glycogen digestion by amyloglucosidase (300 μ l/sample at an activity of 4.5 U/ml in 0.2 M NaAc, pH 4.8). Following an incubation for 2 h at 37°C, the samples were neutralized with 0.25 M NaOH and the glucose concentrations were determined fluorometrically using the Amplex Red reagent (Molecular Probes, Eugene, OR).

Statistical analysis

Results are presented as means \pm standard error of the mean (SEM). For group comparisons the unpaired Student's *t*-test was used. Values of $p < 0.05$ were considered to differ significantly. For all read-outs, a representative or pooled data of minimally three experiments are shown.

RESULTS

Identification and characterization of an inhibitor of the G_L -GP interaction

An inhibitor of the G_L -GP interaction (G_L -GPi) was identified in a miniaturized scintillation proximity assay. This inhibitor suppressed the interaction between a biotin-labelled G_L peptide and recombinant human $GP\alpha$ with an IC_{50} of 0.15 μ M (Table 1). G_L -GPi had no effect on the enzymatic activity of GP at all concentrations tested *in vitro* ($IC_{50} > 30 \mu$ M). Two direct GPis, CP-320,626 [13] and compound A (Bsp.1 in [Aventis Pharma Deutschland GmbH: DE 10335092 B3 (February 3, 2005)]), were used as reference compounds in all experiments. These two compounds inhibited GP activity with IC_{50} 's of 1.4 μ M (CP-320,626) and 2 μ M (compound A). With respect to the G_L -GP interaction, CP-320,626 also suppressed the interaction with an IC_{50} of 1 μ M, whereas compound A did not affect the G_L -GP interaction. G_L -GPi was tested in primary rat hepatocytes using a crystal violet staining [14] and showed no cytotoxic effect at concentrations up to 100 μ M (data not shown). Thus G_L -GPi, an inhibitor of the G_L -GP interaction that showed no inhibition of the enzymatic activity of GP *in vitro* was identified.

G_L -GPi increases glycogen synthesis similarly to direct GPis

Since the prevention of the G_L -GP interaction was predicted to increase glycogen synthesis, the ability of G_L -GPi to increase the rate of glycogen synthesis was tested in primary rat hepatocytes used for this and further experiments as model system. A range of compound concentrations (0.01-10 μ M) was used and the rate of [14 C]glucose incorporation into glycogen was analyzed. G_L -GPi increased the rate of glycogen synthesis in a dose-dependent fashion similarly to both direct GPis (Figure 1) with compound A being the most potent compound (EC_{50} 0.3 μ M), while the potencies of G_L -GPi (EC_{50} 1.3 μ M) and CP-320,626 (EC_{50} 1.8 μ M) were comparable. To work under stable experimental conditions, compound concentrations leading to a plateau effect in the glycogen synthesis assay were used for all subsequent experiments. They were 10 μ M for G_L -GPi and CP-320,626 and 3 μ M for compound A.

It has been shown that the prevention of the G_L -GP interaction increases glycogen synthesis rate.

G_L -GPi elevates glycogen storage but does not prevent glycogen mobilization in contrast to direct GPis

To investigate whether the newly identified inhibitor of the G_L -GP interaction increases glycogen storage, the effect of G_L -GPi on glycogen levels was measured and compared to the effects of direct GPis. At elevated glucose concentrations (10 mM), the glycogen amounts were increased to a similar extent (2.6-fold) by the G_L -GPi and direct GPis after 2 h of treatment (Figure 2A). However at prolonged treatment (6 h), glycogen levels continued to increase with direct GPis but plateaued with G_L -GPi. To further discriminate the effects of a direct inhibition of GP and the G_L -GP inhibition, similar treatments were performed at low glucose concentration (2.5 mM) to mimic the state of hypoglycaemia. Under these conditions, basal glycogen levels declined without compound treatment (Figure 2B), indicating glycogen store mobilization over time. However, with direct GPis, glycogen amounts not only remained at the basal level after 2 h of treatment, but further increased after prolonged treatment (2.5-fold higher than starting point (0 h)). In contrast, in hepatocytes treated with G_L -GPi no increase in glycogen levels but a delayed mobilization of glycogen pools was observed at 2.5 mM glucose.

Thus, the G_L -GP inhibitor increased glycogen storage at elevated glucose levels but did not prevent its mobilization at low glucose levels.

Both G_L -GPi and direct GPis promote the dephosphorylation and activation of GS

The molecular mechanisms underlying the differential effects of G_L -GPi on glycogen levels in comparison to GPis were investigated. First, the effects on the phosphorylation state and the activity of GS were analyzed. Both G_L -GPi as well as two direct GPis significantly reduced the phosphorylation status of GS at both 10 and 2.5 mM glucose concentrations after 2 h of treatment (Figure 3A, B). At elevated glucose levels this effect decreased over time (small but significant decrease in GS phosphorylation after 6 h of treatment), while at low glucose levels the effect on GS phosphorylation was rather short lasting (no significant effect after 6 h of treatment). In order to test whether the dephosphorylation of GS translated into an activation of the enzyme, the enzymatic activity of GS was measured. As expected, GS activity was significantly increased by G_L -GPi similarly to both direct GPis (Figure 3C). At the elevated glucose concentrations, this effect was more pronounced at 2 h (200-250% activity) compared to 6 h (150-170% activity) of compound treatment, indicating that the activity measured reflects the phosphorylation status of GS. A similar pattern in affecting the GS activity was observed at 2.5 mM glucose.

Overall, both direct GP inhibition and the prevention of the G_L -GP interaction led to the dephosphorylation and consequent activation of GS.

G_L -GPi has no effect on the activity of recombinant liver GS

To ensure that the activation of GS in hepatocytes is not attributed to a direct effect of G_L -GPi on the enzyme, the effect of G_L -GPi on the activity of recombinant mouse liver GS was measured. Treatment of GS with the catalytic subunit of recombinant PP1 caused a complete dephosphorylation of GS (Figure 4A). This effect was reduced by 17 mM sodium orthovanadate, a nonspecific protein phosphate inhibitor. Furthermore, treatment with PP1 increased the enzymatic activity of GS at both 0.1 mM glucose 6-phosphate (over 20-fold) (Figure 4B) and 10 mM glucose 6-phosphate (2-fold increase, Figure 4C). As expected, 10 mM glucose 6-phosphate per se had a strong stimulatory effect on GS activity. In contrast, G_L -GPi had no effect on GS activity at the concentrations up to 100 μ M at both 0.1 and 10 mM glucose 6-phosphate.

These data show that the activation of GS in hepatocytes is not caused by a direct effect of G_L -GPi on GS.

Inhibition of the G_L -GP interaction does not inactivate and dephosphorylate cellular GP

To confirm that the prevention of the G_L -GP interaction does not inhibit the cellular activity of GP, the effect of G_L -GPi on the endogenous activity of GP in primary rat hepatocytes was analyzed. The activity of the enzyme measured in the glycogenolytic direction was significantly reduced (by 58-86%) by both direct GPis independent of the glucose concentrations used at both 2 and 6 h (Figure 5A). In contrast, G_L -GPi did not cause any reduction in the enzymatic activity of GP at any of the glucose concentrations or time points tested. Rather a slight, non-significant increase in GP activity at 6 h of treatment was visible. The reduction in GP activity with the direct GPis was associated with a decreased level of GP phosphorylation. A nearly complete abolishment of the GP phosphorylation at serine 14 was observed with the direct GPis at both glucose concentrations after 2 h treatment, slowly recovering thereafter (Figure 5B, C). In contrast, the phosphorylation status of GP was not influenced following a 2 h treatment with the G_L -GP inhibitor at both glucose concentrations, correlating with the unchanged GP activity. At the 6 h time point, it was slightly but non-significantly increased, reflecting a possible trend towards an activation of GP.

Overall, in contrast to direct GPis, the inhibition of the G_L -GP interaction does not reduce neither the cellular activity of GP nor its phosphorylation over 6 h.

Direct GPis but not G_L -GPi reduce the amounts of GP in the glycogen fraction

Since changes in the subcellular compartmentalization of key enzymes is one of the mechanisms involved in the regulation of glycogen metabolism [15], the impact of G_L -GPi and direct GPis on the amounts of GP found in the glycogen-enriched fraction was analyzed. In the immunoblotted samples normalized to the protein content, significant differences in GP signals were observed with profound reduction in GP amounts following the treatment with direct GPis compared to the treatment with G_L -GPi as the most prominent feature (Figure 6A). To assess whether the stoichiometry of GP-glycogen binding was altered upon compound action, the relative amounts of GP were normalized to the glycogen content of the same samples. The GP/glycogen ratios were significantly reduced (by 80-90%) by both GPis but only moderately (by 36%) by G_L -GPi at 10 mM glucose compared to DMSO control at 0 h time point (Figure 6B). At 2.5 mM glucose, the GP/glycogen ratios remained unchanged following the treatment with G_L -GPi and were again significantly reduced by direct GPis (by 70-85%).

These data show that in contrast to G_L -GPi, the treatment with direct GP inhibitors pronouncedly displaces GP from glycogen particles, thereby decreasing the ratio of GP per glycogen.

4. DISCUSSION

It has been hypothesized that a drug blocking the G_L -GP interaction would activate GS and increase glycogen synthesis, and could thereby potentially attenuate the state of diabetic hyperglycaemia [9]. For the first time, we describe here the characterization of an inhibitor of the G_L -GP interaction.

This inhibitor suppressed the interaction between the G_L peptide and GP_a with an IC_{50} of 150 nM. Importantly, it did not inhibit the enzymatic activity of GP. These data suggest that displacement of G_L from GP does not cause significant conformational changes that would reduce the activity of GP. This is in agreement with our recent x-ray crystallographic study showing no large conformational differences between h GP_a crystallized alone or in a complex with a G_L peptide [16].

The direct GP inhibitor CP-320,626 also caused an inhibitory effect on the interaction between the G_L and GP_a with an IC_{50} of 1 μ M, which is in the same concentration range as the IC_{50} for GP_a inhibition (IC_{50} 1.4 μ M). As has been shown recently, a related compound CP-316,819 is also capable of blocking the G_L -GP interaction [17]. Both compounds are members of the indole-2-carboxamide series of phosphorylase inhibitors [18]. The G_L protein binds to the AMP binding site of GP [16], which is distinct from the indole-binding site on GP. Therefore, the effect of these compounds on the G_L -GP interaction cannot be explained by an overlap of these two sites. More likely, these compounds induce conformational changes in the GP protein, which prevents its binding to G_L . The compound A, another potent GPi that belongs to the class of acyl ureas, did not affect the G_L -GP interaction in the present study despite the fact that compounds of this class have been shown to bind to the AMP binding site of GP [19]. This indicates differences in binding to this area of the GP protein by G_L and compound A.

It was previously reported that in hepatocytes, the action of CP-320,626 was associated with the conversion of GP from phosphorylated *a* to dephosphorylated *b* form [20]. In the present study we confirmed this effect of CP-320,626 and showed that the action of a member of acyl urea class of GP inhibitors, compound A, was also associated with the dephosphorylation of GP. In contrast, G_L -GPi did not lead to a GP dephosphorylation at 2 h, which correlated with the unchanged GP activity. At 6 h, a slight increase in GP phosphorylation was observed, which explains the trend towards an increased GP activity after a prolonged treatment using the G_L -GPi. Based on this trend, we cannot rule out a possible feed-back regulation that might be switched on in order to counteract a sustained stimulation of glycogen synthesis. Further studies should be undertaken to clarify this phenomenon.

In hepatocytes, the inactivation of GP by CP-320,626 led to an increase in glycogen synthesis [21, 22]. In the present study we provide the first pharmacological evidence demonstrating that prevention of the G_L -GP interaction is sufficient to increase glycogen synthesis in hepatocytes without inhibiting the activity of GP. G_L -GPi consistently increased GS activity similarly to both direct GPis, which correlated well with a reduced GS phosphorylation at most conditions. We did not see this correlation at 6 h of treatment at low glucose where no decrease in GS phosphorylation was measurable. A possible explanation for this discrepancy could be a lower sensitivity of the GS phosphoblot compared to radioactive method of GS activity determination. G_L -GPi increased the rate of glycogen synthesis similarly to direct GPis. Since G_L -GPi did not have any effect on the activity of recombinant GS, the increases in GS activity and glycogen synthesis rate caused by G_L -GPi can be entirely attributed to the inhibition of the G_L -GP interaction.

Modulation of the subcellular compartmentalization of key enzymes is one of the mechanisms involved in the regulation of glycogen metabolism [15]. Therefore, we have investigated the impact of the compounds on the association of GP with the glycogen-enriched fractions. Direct GPis significantly reduced the amounts of GP present in glycogen-enriched fractions compared to the treatment with G_L -GPi. In addition, direct GPis profoundly reduced the GP/glycogen ratios at both 10 mM and 2.5 mM glucose concentrations. In contrast, the G_L -

GP inhibitor only moderately reduced the GP/glycogen ratio at 10 mM glucose, possibly due to a saturation of the GP/glycogen binding at high glycogen levels, and did not change the GP/glycogen ratio at 2.5 mM glucose. These data demonstrate that the effects of G_L -GPi and direct GPis on the stoichiometry of GP/glycogen binding are different with a pronounced and consistent displacement of GP from the glycogen particles by direct GPis. A possible explanation for the effect of direct GPis could be a weaker binding of GP to glycogen particles due to the loss of phosphorylation/activity.

We show here for the first time that a pharmacological interference with the G_L -GP interaction is sufficient to increase glycogen levels without inhibiting the activity of GP. G_L -GPi increased glycogen levels with an efficacy similar to direct GPis following 2 h treatment at 10 mM glucose. Prolonged treatment up to 6 h with G_L -GPi did not lead to a further increase in glycogen levels. This observation could be explained by a futile glycogenolysis-glycogen synthesis cycle that occurs due to the co-existence of the activated forms of both glycogen phosphorylase and glycogen synthase [23, 24]. The higher efficacy towards elevating glycogen levels demonstrated by direct GPis at 6 h can be attributed to an increase in glycogen synthesis and a simultaneous prevention of glycogenolysis. A reduction of GP within the glycogen-enriched fraction upon treatment with direct GPis, as demonstrated here, could additionally potentiate the action of these compounds, for example by a delayed recruitment of GP to the glycogen particles as a site of action.

At 2.5 mM glucose, G_L -GPi did not lead to glycogen accumulation and rather allowed its mobilization over time, indicating that at low glucose levels the natural regulation of glycogen metabolism favouring glycogenolysis is functional in the presence of the G_L -GPi. The ability of direct GPis to increase glycogen levels with high efficacy even at 2.5 mM glucose is explained by their glucose-independent effects on GP activity and phosphorylation. This glucose independence observed with CP-320,626 is in line with a previous report showing that this compound inhibited liver GP *in vitro* and in primary rat hepatocytes independent of the glucose concentration [25].

In this study we show that G_L -GPi does not prevent the mobilization of glycogen because the activity of GP is not impaired. Based on this observation it is tempting to speculate that the risk of hypoglycaemia upon an inhibition of the G_L -GP interaction could be lower compared to an inhibition of the GP enzyme. In addition, associated with a direct inhibition of GP are potential side effects in muscle. It has been shown that a pharmacological inhibition of GP impairs the aerobic muscle function during prolonged contraction [6]. Furthermore, genetic deficiency in muscle glycogen phosphorylase, known as glycogenosis type V or McArdle's disease [26], significantly reduces the exercise capacity in these patients. Because G_L protein is also expressed in human muscle [27], G_L -GP inhibitors could also be functional in this tissue. But since the inhibition of the G_L -GP interaction allows glycogen mobilization, the detrimental effects in muscle are not foreseen for G_L -GP inhibitors. Therefore, a compound working via this novel mechanism could potentially offer a better risk-benefit profile, however at this stage it is too early to predict whether this will provide clinical advantages.

In summary, we have identified an inhibitor of the G_L -GP interaction and provide first evidence that the pharmacological prevention of the G_L -GP interaction increases both glycogen synthesis and glycogen accumulation in primary rat hepatocytes. In contrast to direct GP inhibitors, these effects were achieved without inhibiting GP activity. Taken together, our study suggests that the interaction between G_L and GP plays an important role in the regulation of the glycogen metabolism and supports the hypothesis that the prevention of this interaction might be an attractive novel anti-diabetic strategy.

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FIGURE LEGENDS

Figure 1 Both G_L-GPi and direct GPis increase glycogen synthesis in a dose-dependent manner

Primary rat hepatocytes were cultured in Krebs-Ringer-bicarbonate/HEPES buffer containing 10 mM glucose and treated with compounds used in a range of concentrations for 2 h in the presence of [¹⁴C]glucose. The rate of glycogen synthesis was determined from the activity of [¹⁴C]glucose incorporated into glycogen. A representative of minimally three experiments is shown.

Figure 2 G_L-GPi modulates the storage of glycogen in a glucose-dependent manner

After overnight incubation at 5 mM glucose, primary rat hepatocytes were treated for 2 or 6 h with 10 μM G_L-GPi, 3 μM compound A, 10 μM CP-320,626 or 0.25% DMSO (solvent control) at 10 mM (A) or 2.5 mM (B) glucose, and glycogen amounts were determined. Glycogen levels at 0 h time point represent the glycogen content prior to the compound application. Glycogen amounts are expressed as nmol glucose equivalents per mg protein content in the glycogen-enriched fraction used for the assay. Values are presented as means±SEM of four independent experiments performed in duplicate. Significances of differences are: ** p<0.01 and *** p<0.001.

Figure 3 Dephosphorylation and activation of glycogen synthase by G_L-GPi and GPis

Primary rat hepatocytes were treated for 2 or 6 h with G_L-GPi (10 μM), compound A (3 μM), CP-320,626 (10 μM) or 0.25% DMSO at 10 mM or 2.5 mM glucose. Cells were harvested and the lysates were used for the determination of GS phosphorylation (A, B) and GS activity (C). The phosphorylation status was determined using anti-phospho(Ser⁶⁴¹)-GS antibody and anti-GS antibody as an internal control. (B) Densitometric analysis of three phosphoblots. (C) Glycogen synthase activity. Data are expressed as the activity ratio defined as the ratio of the activity measured at 0.1 mM glucose 6-phosphate relative to activity in the presence of 10 mM glucose 6-phosphate. Values are presented as means±SEM of three experiments, *p<0.05 vs. respective vehicle controls.

Figure 4 G_L-GPi does not influence enzymatic activity of GS

Recombinant mouse liver GS was treated with PP1 (25 U/ml) in the presence or absence of 17 mM sodium orthovanadate for 30 minutes at 30°C followed by determination of phosphorylation state of the enzyme using anti-phospho(Ser641)-GS antibody and anti-GS antibody as an internal control (A). In separate experiments, recombinant GS protein was treated with G_L-GPi (0.3-100 μM) or catalytic subunit of PP1 (147 U/ml) as a positive control for 30 minutes at 30°C followed by the determination of GS activity at 0.1 and 10 mM glucose 6-phosphate (B, C). The activity of the GS was determined from the activity of [¹⁴C]UDP-glucose incorporated into glycogen. Significances of differences are: ** p<0.01 and *** p<0.001.

Figure 5 Inactivation (A) and dephosphorylation (B, C) of glycogen phosphorylase by direct GPis but not G_L-GPi

Primary rat hepatocytes were treated for 2 or 6 h with 10 μM G_L-GPi, 3 μM compound A, 10 μM CP-320,626 or 0.25% DMSO at 10 mM or 2.5 mM glucose. Cell lysates were analyzed with respect to GP activity (A) and GP phosphorylation (B, C) using anti-phospho(Ser¹⁴)-GP antibody (upper panel); anti-GP antibody ((B), bottom panel) were used as internal control. Values of GP activity (A) and densitometric analysis of phosphoblots (C) are presented as means±SEM for three experiments, * p<0.05 vs. respective vehicle controls.

Figure 6 Direct GPis but not G_L -GPi reduce amounts of GP in glycogen fraction

Glycogen-enriched fractions obtained by differential centrifugation were analyzed by immunoblotting (A) with anti-GP antibody after the incubation of hepatocytes for 2 or 6 h with 10 μ M G_L -GPi, 3 μ M compound A, 10 μ M CP-320,626 or 0.25% DMSO at 10 mM or 2.5 mM glucose. Immunoblot analysis with anti- γ -tubulin antibody (A, bottom panel) using the same samples served as an internal control. (B) Relative amounts of GP in the glycogen-enriched fractions corrected to the glycogen amounts in the same samples (GP/glycogen ratio). Values are presented as means \pm SEM of four independent experiments, * $p < 0.05$ vs. respective vehicle control at 0h time point (state prior to the application of compounds), # $p < 0.05$ vs. respective treatments with G_L -GPi.

Table 1. Inhibition of G_L -GPa interaction by G_L -GPi and recombinant human liver GPa by direct GPis

Interaction between biotin-labelled G_L peptide and recombinant human liver [33 P]radiolabelled GPa was assessed by a scintillation proximity assay (SPA). Human liver GPa activity was measured in the direction of glycogenolysis. The IC_{50} values given are means of pooled data from 4-6 experiments.

Compound	G_L -GPa interaction (SPA assay), IC_{50} , μ M	Human liver GPa IC_{50} , μ M
G_L -GPi	0.15	> 30
CP-320,626	1	1.4
Compound A	> 30	2

Figure 1

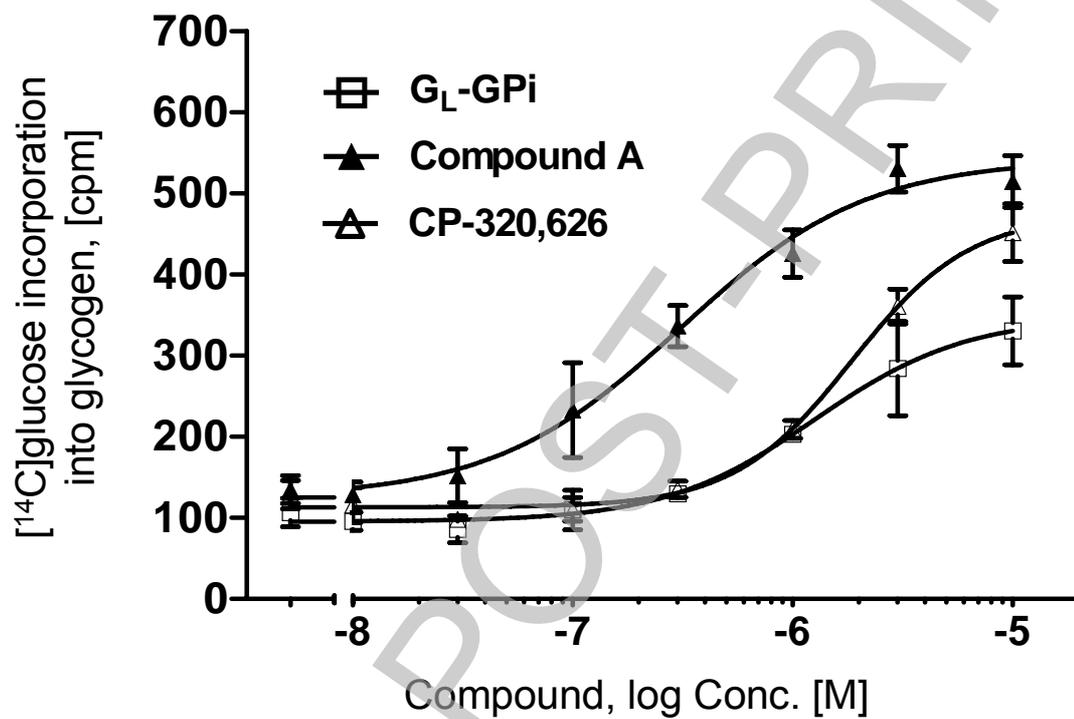
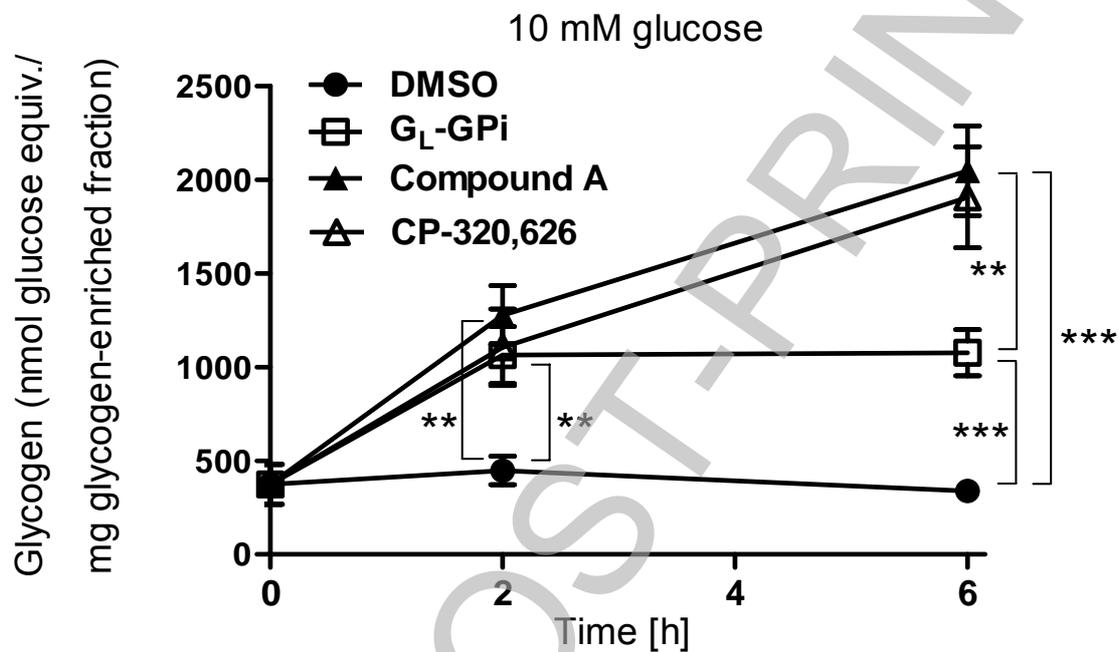


Figure 2

A



B

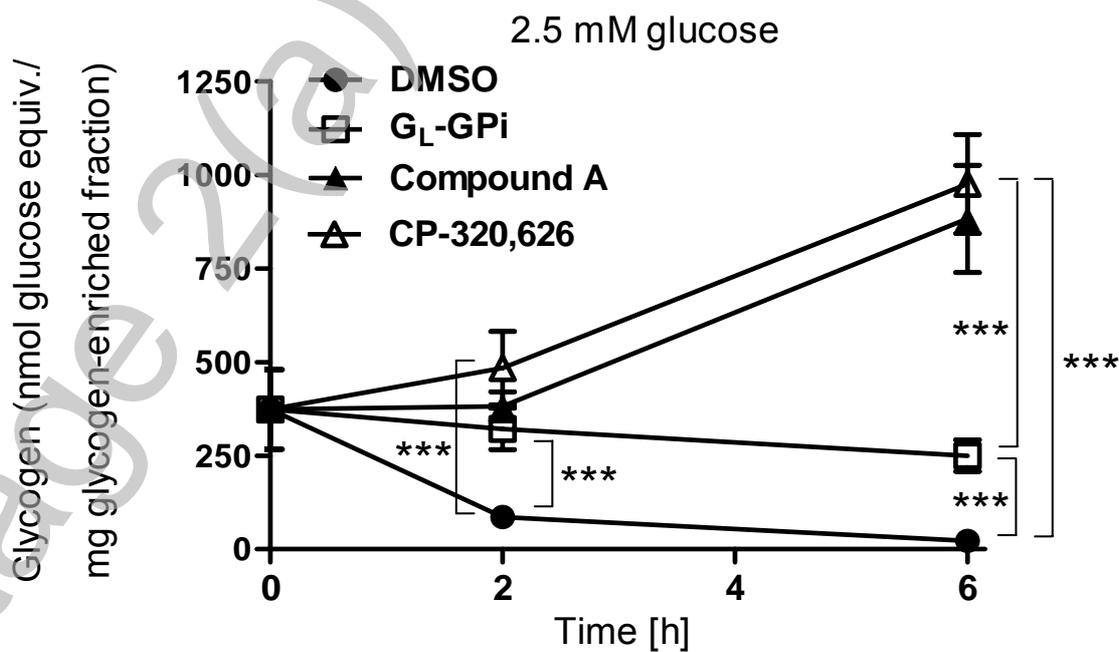
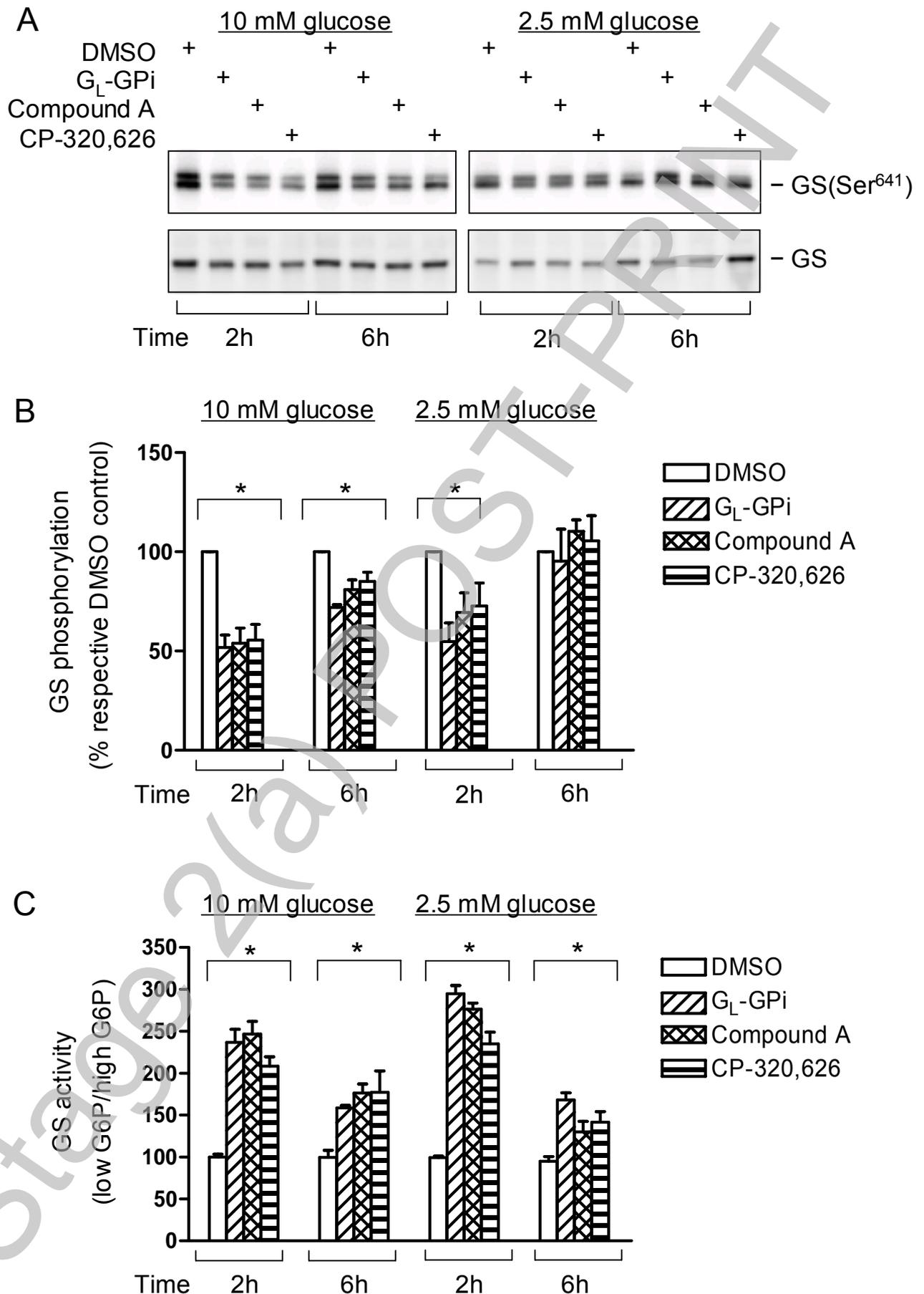


Figure 3



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Figure 4

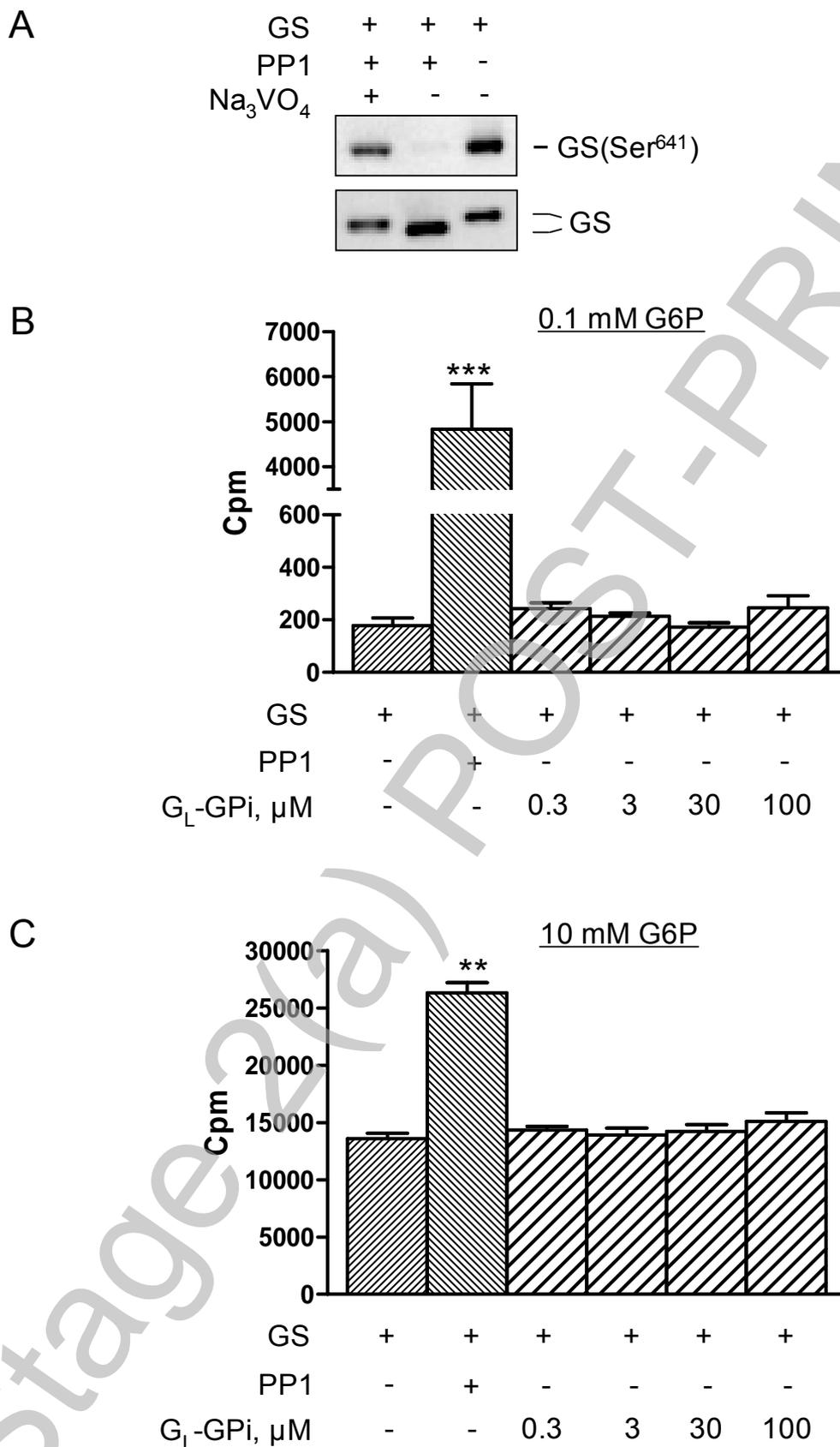
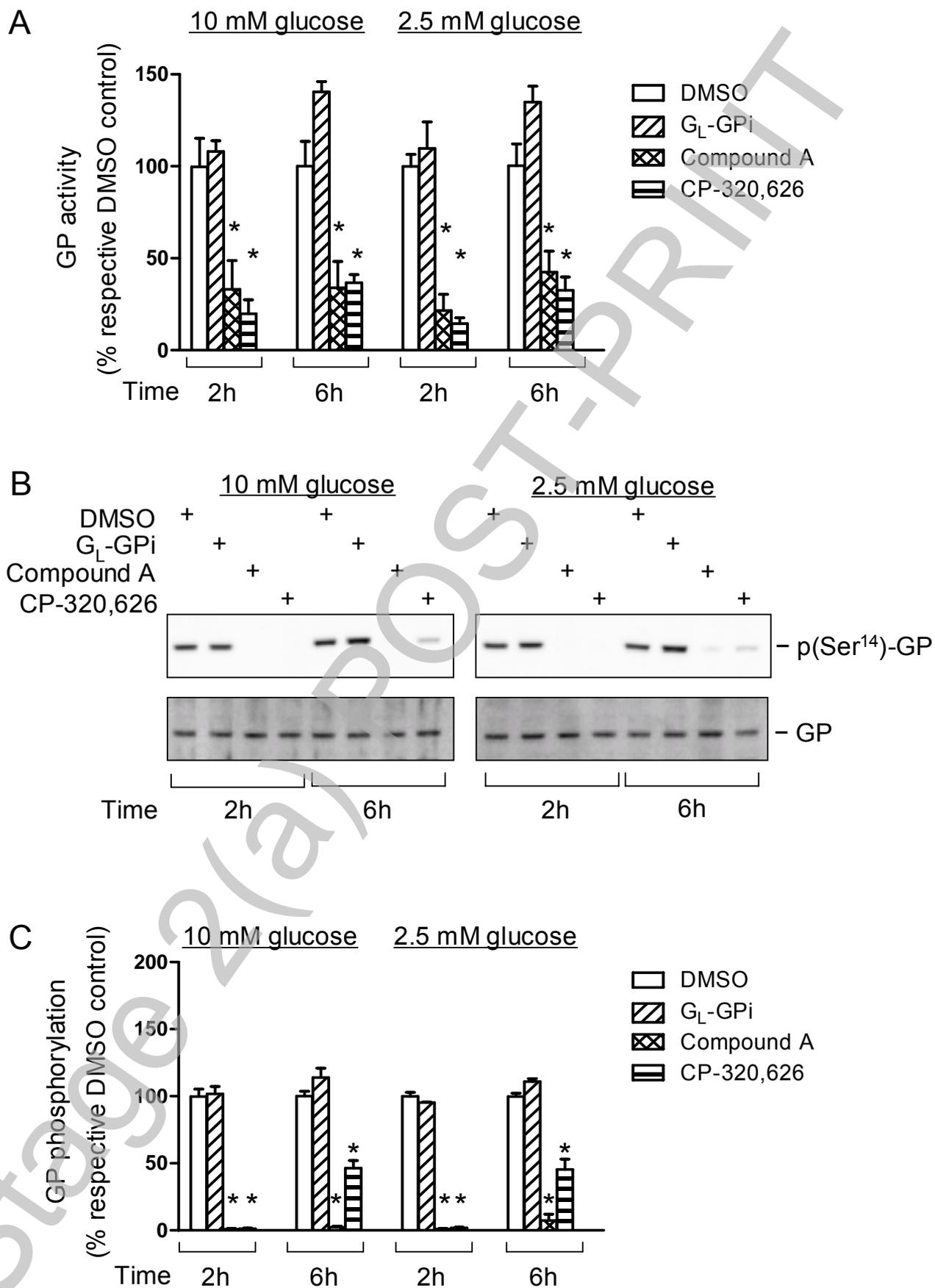


Figure 5



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Figure 6

